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(57) Abstract

The present invention relates to recombinant expression vectors carrying a gene encoding a therapeutically active polypeptide, which gene is under transcriptional control of a ubiquitin promoter. The invention also relates to the use of a ubiquitin promoter to direct *in vivo* expression of therapeutic genes after transfer of such genes to the central nervous system.

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EXPRESSION VECTORS AND METHODS FOR *IN VIVO* EXPRESSION OF THERAPEUTIC POLYPEPTIDES

TECHNICAL FIELD

The present invention relates to recombinant expression vectors carrying a gene encoding a therapeutically active polypeptide, which gene is under transcriptional control of a ubiquitin promoter. The invention also relates to the use of a ubiquitin promoter to direct *in vivo* expression of therapeutic genes after transfer of such genes to the central nervous system.

BACKGROUND ART

Therapeutically active polypeptides may be delivered to the central nervous system (CNS) by implantation of polypeptide producing cells, or by injection of viral vectors capable of infecting brain cells and directing the expression of a therapeutically active polypeptide encoded by the viral vector in the brain cells.

Various ways of achieving therapy of the CNS using intracerebral implantation of genetically modified cells and the use of viral vectors for the delivery of therapeutic genes to the CNS has been considered.

According to one approach, primary and immortalised cells have been successfully used for gene transfer applications. Examples of such cells are primary cells of neuronal origin (e.g. glia cells and astrocytes), or of non-neuronal origin (e.g. fibroblasts, myoblasts and hepatocytes). Such cells may not survive for longer periods in the CNS unless they are immortalised. Intracerebral grafting of foetal tissue for the treatment of neurological disorders have also been investigated. Alternatively stem cells may be used.

Another cell type which has been proposed as gene transfer vehicle is the cerebral endothelial cell. The direct implantation of immortalised and genetically transformed cerebral endothelial cells into various parts of the brain has been disclosed. It has also been suggested to deliver therapeutic agents by infecting endothelial cells of blood vessels located in the brain with a viral vector, as a result of

intravascular administration of the vector to the host near the site of infection (WO 96/22112).

Alternatively, a broad range of viral vectors such as including adenovirus vectors (WO 95/26408), adeno-associated virus vectors (WO 95/34670), herpes virus vectors (*Glorioso et al.* Sem. Virol. 1992 **3** 265-276), vaccinia virus vectors, and retroviral vectors, including systems based on HIV, has been suggested as delivery vehicles for therapeutic genes to the CNS. Such vectors can be administered to the CNS by the intravenous and the intracranial route, and by administration to the cerebrospinal fluid.

Thus WO 95/09654 discloses a method for the treatment of adverse conditions of the CNS by administration of producer cells containing a retroviral vector to the cerebrospinal fluid. The producer cells produce retroviral particles which is capable of transducing cells present in the nervous system.

Intracerebral implantation of encapsulated cells producing a therapeutic peptide has also been suggested.

The vast majority of *in vivo* gene transfer experiments in the mammalian brain have used constitutively expressed viral promoters. Such promoters have a major advantage in terms of transcriptional strength and activity in most cell types. There are, however, significant disadvantages of such promoters. Despite the persistance of vector DNA, a loss of expression over time has been observed.

Ubiquitin proteins are "house hold proteins", essential for the catabolism of all cellular proteins. The structure of ubiquitin proteins is highly conserved during evolution.

The ubiquitin promoter is a constitutive promoter which controls the expression of ubiquitin proteins. A number of transfection experiments have shown that this promoter is as strong as, or even stronger than the commonly used viral promoters. Furthermore, heterologous genes under the control of the ubiquitin promoter are actively transcribed in every cell line that has been transfected with this promoter (more than 20, including several cell lines of neuronal origin). In transgenic mice, the human ubiquitin C promoter (UbC) have been shown to direct *in vivo* expression of heterologous genes in several types of tissues, including brain-tissue (Nucleic Acids Research 1996 **24** (9) 1787-1788).

In order to obtain efficient expression in the CNS, the therapeutic gene must be put under transcriptional control of a promoter which is active in the CNS. However, viral promoters are down-regulated *in vivo* in mammalian cells. If efficient transfer of a therapeutic polypeptide to the CNS is to be obtained with a viral vector, it is should be replaced the with a promoter which will provide stable and efficient expression.

As described above, the ubiquitin promoter has proven to direct stable high level expression of heterologous genes in *in vitro* experiments. However, there is no indication of its efficiency *in vivo*.

SUMMARY OF THE INVENTION

The present invention is directed to the use of an ubiquitin promoter for stable and efficient expression of genes encoding therapeutically active polypeptides in the CNS. An ubiquitin promoter of present invention includes the human ubiquitin promoter, which is indistinguishable from the endogenous ubiquitin promoter and should therefore not be susceptible to the *in vivo* down-regulation observed for viral promoters.

Accordingly, in its first aspect, the present invention is directed towards a recombinant expression vector comprising a gene encoding a therapeutically active polypeptide, which gene is under transcriptional control of an ubiquitin promoter.

In its second aspect, the invention provides an eukaryotic cell, transfected with the eukaryotic expression vector of the invention.

In a third aspect, the invention provides a packaging cell line comprising the retroviral expression vector of the invention and one or more nucleotide constructs encoding the proteins required for the genome of the retroviral vector to be packaged.

In a fourth aspect, the invention provides a method of producing a retroviral particle by culturing the packaging cell line of the invention, and a retroviral particle obtained according to this method.

In a fourth aspect, the invention provides an eukaryotic cell, e.g. an immortalised cerebral endothelial cell line, infected by a retroviral particle obtained by the method of this invention.

In a sixth aspect, the invention is directed towards the use of the recombinant expression vector of the invention for the manufacture of a pharmaceutical composition, useful for the treatment of a neurological disease or disorder.

In a seventh aspect, the invention provides a method for the treatment of a living body suffering from a neurological disease or disorder which is responsive to a therapeutically active polypeptide, which method comprises administering to the living body, a retroviral particle obtained by the method of the invention, encoding the therapeutically active polypeptide; or implanting into the living body, the cells of the invention, producing the therapeutically active polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is further illustrated by reference to the accompanying drawing in which:

Fig. 1 shows a graphic illustration of a UbC promoter containing plasmid based expression vector, pUbi1z, obtained according to Example 2.

DETAILED DISCLOSURE OF THE INVENTION

Recombinant Expression Vectors

The present invention resides in the use of a ubiquitin promoter to direct *in vivo* expression of therapeutic genes after transfer of such genes to the central nervous system.

Accordingly, in its first aspect the invention provides a recombinant expression vector comprising a gene encoding a therapeutically active polypeptide, which gene is under transcriptional control of an ubiquitin promoter.

The recombinant expression vector of the invention may be any vector suitable for transferring a gene under transcriptional control of a ubiquitin promoter to mammalian cells. However, in a preferred embodiment, the recombinant expression vector is an eukaryotic expression vector or a recombinant viral expression vector.

Genes can be transferred into cells using a variety of means including calcium phosphate precipitation [*Graham et al.*, Virol. 1973 **52** 456-467; *Wigler et al.*, Cell 1979 777-785], electroporation [*Neumann et al.*, EMBO J. 1982 841-845], microinjection [*Graessmann et al.*, Meth. Enzymology 1983 **101** 482-492], by means of liposomes [*Staubinger et al.*, Methods in Enzymology 1083 **101** 512-527], spheroblasts [*Schaffner et al.*, Proc. Natl. Acad. Sci. USA, 1980 **77** 2163-2167], by means of recombinant viruses, or by other methods known to those skilled in the art.

Viral Expression Vectors

Viral vectors provide an efficient means of transferring genes into cells both *in vivo* and in vitro. The efficiency of viral gene-transfer is due to the fact that transfer of DNA is an essential part of the natural life cycle of viruses and that DNA transfer is a receptor-mediated process. Several viral systems including retrovirus, adenovirus, adeno-associated virus, vaccinia virus and herpes virus have been developed as *in vivo* therapeutic gene transfer vectors for gene therapy of CNS disorders.

Viral vectors have also been used to transform various forms of cells such as astrocytes, fibroblast cells and cerebral endothelial cells which is thereafter implanted into the CNS. Further WO 96/06942 describe genetically altered T-cells which enter the CNS and may be used as gene transfer vehicles.

The recombinant viral expression vector of the invention may be any viral expression vector suited for *in vivo* transfer and expression of genes. Preferred viral expression vectors include retroviral vectors, recombinant adenovirus vectors, recombinant adeno-associated virus vectors, vaccinia virus vectors and recombinant herpes virus vectors.

Recombinant retroviral gene delivery methods are the most extensively used due in part to: (1) the efficient entry of genetic material (the vector genome) into replicating cells; (2) an active, efficient process of entry into the target cell nucleus; (3) relatively high levels of gene expression; (4) the potential to target particular cellular subtypes through control of the vector target cell binding and the tissue specific control of gene expression; (5) a general lack of pre-existing host immunity; and substantial knowledge and clinical experience which has been gained with such vectors.

In a preferred embodiment of the invention, the recombinant vector carrying a therapeutic gene under transcriptional control of an ubiquitin promoter is therefore a retroviral vector.

The retroviral genome consists of an RNA molecule with the structure R-U5-gag-pol-env-U3-R. During the process of reverse transcription, the U5 region is duplicated and placed at the right hand end of the generated DNA molecule, whilst the U3 region is duplicated and placed at the left hand end of the generated DNA molecule. The resulting terminal structure U3-R-U5 is called LTR (Long Terminal Repeat) and is thus identical and repeated at both ends of the DNA structure or provirus. The U3 region at the left hand end of the provirus harbours the promoter that is used to drive expression after infection has occurred. This promoter drives the synthesis of an RNA transcript initiating at the boundary between the left hand U3 and R regions and terminating at the boundary between the right hand R and U5 region. The RNA is packaged into retroviral particles and transported into the target cell to be infected. In the target cell the RNA genome is reverse transcribed as described above.

Retroviral vector systems used for the generation of recombinant retroviral particles consist of two components.

The retroviral vector itself is a modified retrovirus (vector plasmid) in which the genes encoding for the viral proteins (gag, pol and/or env) have been replaced by therapeutic genes and/or marker genes to be transferred to the target cell. Since the replacement of the genes encoding for the viral proteins effectively cripples the virus it must be rescued by the second component in the system which provides the missing viral proteins to the modified retrovirus.

The second component is a cell line that produces large quantities of the viral proteins (gag, pol and/or env), however lacks the ability to produce replication competent virus. This cell line is known as the packaging cell line and consists of a cell line transfected with one or more plasmids carrying the genes (gag, pol and/or env) enabling the modified retroviral vector to be packaged.

To generate the packaged vector, the vector plasmid is transfected into the packaging cell line. Under these conditions the modified retroviral genome including the inserted therapeutic and marker genes is transcribed from the vector plasmid and packaged into modified retroviral particles (recombinant viral particles). This recombinant virus is then used to infect target cells in vitro or *in vivo* and the vector

genome and any carried marker or therapeutic genes become integrated into the target cell's DNA. A cell infected with such a recombinant viral particle cannot produce new vector virus since no viral proteins are present in these cells. However the DNA of the vector carrying the therapeutic and marker genes is integrated in the cell's DNA as a provirus and can now be expressed in the infected cell.

WO-A1-9607748 describes the principle and construction of a new type of retroviral vector. In the ProCon-vector plasmid the right-hand (3') U3 region is altered, but the normal left-hand (5') U3 structure is maintained; the vector can be normally transcribed into RNA utilising the normal retroviral promoter located within the left-hand (5') U3 region upon its introduction into packaging cells. However the generated RNA will only contain the altered right-hand (3') U3 structure. In the infected target cell, after reverse transcription, this altered U3 structure will be present in both Long Terminal Repeat at either end of the retroviral structure.

If the altered region carries a polylinker instead of the U3 region (which contain the viral promoter) then any promoter, including the human ubiquitin promoter, can be inserted and this promoter is then utilised exclusively in the target cell for expression of linked sequences encoding therapeutic polypeptides.

DNA segments homologous to one or more cellular sequences can also be inserted into the polylinker for the purposes of gene targeting, by homologous recombination.

The retroviral vectors of the invention need not be of the ProCon type, but can be any conventional retroviral vector carrying therapeutic genes under transcriptional control of the an ubiquitin promoter.

Such vectors include Self-Inactivating-Vectors (SIN) in which retroviral promoters are functionally inactivated in the target cell (WO-A1-94/29437). Further modifications of these vectors include the insertion of promoter gene cassettes within the LTR region to create double copy vectors (WO-A1-89/11539). In both of these vectors the heterologous promoters inserted either in the body of the vector, or in the LTR region are directly linked to the therapeutic gene.

The retroviral vectors of the invention are based preferably either on a BAG vector [*Price, Turner J D, and Cepko C*; Proc. Natl. Acad. Sci. USA 1987 **84** 156-160) or an LXSN vector [*Miller A D, & Rossman G J*; Biotechniques 1989 **7** 980-990].

Eukaryotic Expression Vectors

The recombinant eukaryotic expression vector of the invention may be any eukaryotic expression vector suited for transferring a gene under transcriptional control of a ubiquitin promoter to mammalian cells.

Preferred eukaryotic expression vector of the invention are pTEJ-4, pTEJ-8, or pUbi1Z.

As eukaryotic expression vectors have to be propagated in bacteria prior to transfection of eukaryotic cells, the eukaryotic expression vector should contain sequences which facilitate the prokaryotic propagation along with two eukaryotic transcription units.

Prokaryotic sequences include a bacterial resistance gene under the transcriptional control of the prokaryotic EM7 promoter and a bacterial origin of DNA replication. The eukaryotic transcription unit responsible for eukaryotic selection employ the SV 40 early promoter to drive the expression of a resistance gene and a polyadenylation signal.

The present expression vectors contain one of the following prokaryotic/eukaryotic selection markers: neomycin, hygromycin, pyromycin or zeocin resistance gene allowing the selection of bacterial clones under EM7 promoter and cellular clones under SV 40 early promoter. The second eukaryotic transcription unit contain the UbC promoter, a polyadenylation signal, and finally a polylinker for insertion of nucleotide sequences encoding the protein in question (as an example see pUbi1Z, of Example 2).

Ubiquitin Promoters

The ubiquitin promoter contemplated according to the present invention is an ubiquitin promoter derived from any convenient origin, e.g. derived from the 5' end (upstream) of the ubiquitin gene. Preferably the ubiquitin promoter is of human origin, in particular the human ubiquitin C promoter (UbC).

In its most preferred embodiment, the ubiquitin promoter of the invention is a human ubiquitin promoter having the sequence presented as SEQ ID NO: 1 in the attached sequence listing.

Therapeutically Active Polypeptides

A therapeutically active polypeptide of the invention may be any peptide, polypeptide or protein that is capable of ameliorating neurological disorders.

In a preferred embodiment the therapeutically active polypeptide of the invention is a Nerve Growth Factor (NGF); a Fibroblast Growth Factor (FGF), in particular an acidic or a basic Fibroblast Growth Factor (aFGF or bFGF); an Insulin-like Growth Factor, in particular IGF I or IGF II; an Endothelial Growth Factor (EGF), in particular a Vascular Endothelial Growth and Permeability Factor (VEGPF); a member of the Transforming Growth Factor (TGF) superfamily, including a Transforming Growth Factor-α and -β (TGFα and TGFβ); a Glial Derived Neurotrophic Factor (GDNF); a Ciliary Neurotrophic Factor (CNTF); a Brain Derived Neurotrophic Factor (BDNF); a Neurotrophin, in particular Neurotrophin 3, 4/5, 6 or 7; a Neuturin (NTN); a Percipin; a Tumor Necrosis Factor (TNF), in particular TNF-α; an interferon, in particular Interferon-α, Interferon-β or Interferon-γ; an interleukin (IL), in particular IL-1, IL-1β, GMCSF, and IL-2 to IL-14; a choline acetyl transferase; a Tyrosine Hydroxylase (TH); a tyrosine decarboxylase; an NT-3 or NT-4; a Super Oxide Dismutase (SOD); an Ornithine Transcarbamylase (OTC); a neurotransmitter; a neuromodulator; a neurohormone; a neurotropic factor; a neuroprotective agent; a negative selective marker such as viral a thymidine kinase, in particular Herpex Simplex thymidine kinase, a cytomegalovirus thymidine kinase, a varicella-zoster virus thymidine kinase or a cytosine deamidase.

The therapeutically active polypeptide of the invention may be employed in order to treat diseases or disorders in the brain and central nervous system. Such diseases and disorders include ischemic strokes; angiogenesis; metabolic diseases of the brain; axonal injury; spinal cord injury; Alzheimer's disease; Amyothropic Lateral Sclerosis (ALS); Parkinson's disease; Huntington's disease; motor neuron diseases; central nervous system infections; epilepsy; post polio syndrome; mucopolysaccharidoses (MPS), in particular MPS types I to VII; lipidoses; in particular Gaucher's disease; Lesch-Nyhan syndrome; X-linked ADL; metachromatic leukodystrophy; Krabbe's disease; Charcot-Marie-Tooth disease; Fragile X; epilepsies; Down's syndrome; phenylketonuria; degenerative disorders; and mental disorders.

The eukaryotic expression vectors and the recombinant retroviral vectors according to the invention may in addition to the therapeutic gene carry a gene

encoding a marker. The marker may in particular be proteins like ß-galactosidase, neomycin, alcohol dehydrogenase, luciferace, puromycin, hypoxanthine phosphoribosyl transferase (HPRT), hygromycin, secreted alkaline phosphatase, or green or blue fluoroscent proteins (GFP).

Recipient Cells

In order to deliver the therapeutically active polypeptide to the CNS, the vector of the invention may be used to transduce cells to secrete the therapeutic polypeptide *in vivo* after being implanted into the CNS.

If cells producing the therapeutically active polypeptide are generated by transfection with a plasmid vector, several recipient cells may be used, e.g. immortalised neural stem cells, immortalised cerebral endothelial cell, or other immortalised cells compatible with the CNS.

If cells capable of secreting therapeutic virus particles are generated by transfection with a viral vector are used for transplantation of the packaging cell, several cells may be used, e.g. immortalised neural stem cells, immortalised cerebral endothelial cell, or other immortalised cell compatible with CNS.

If therapeutic virus particles generated by transfecting a packaging cell with a viral vector should be used for direct injection into the CNS, a large number of human cells may be used, e.g. immortalised neural stem cells, immortalised cerebral endothelial cell, or fibroblast-like human cell, e.g. HEK 293 or HeLa

The packaging cell line of the invention can be selected from an element of the group consisting of psi-2 [*Mann R, Mulligan R C, & Buttimore D*; Cell 1983 **33** 153-159], psi-Crip [*Danos O & Mulligan R C*; Proc. Natl. Acad. Sci. USA 1988 85, 6460-6464], psi-AM [*Cone R D, & Mulligan R C*, Proc. Natl. Acad. Sci. USA, 1984 **81** 6349-6353], GP+E-86 [*Markowitz D, Goff S, & Bank A*; J. Virol. 1988 **62** 1120-1124], PA317 [*Miller A D, & Buttimore C*; Mol. Cell. Biol. 1986 **6** 2895-2902], GP+envAM-12 [*Markowitz D, Goff S, & Bank A*; Virology 1988 **167** 400-406], Bosc 23, Bing [*Pear W S, Nolan G P, Scott M L, & Buttimore D*; Proc. Natl. Acad. Sci. USA, 1993 **90** 8392-8396] or FLYA13, FLYRD18 [*Cosset F L, Takeuchi Y, Battini J L, Weiss R A, & Collins M K L*; J. Virol. 1995 **69** 74309-7436], or of any of these transfected with recombinant constructs allowing expression of surface proteins from other enveloped viruses. Such pseudotyped retroviral particles are described in PCT/EP96/01348.

In a particular preferred embodiment, the packaging cell line is made from human cells, e.g. HT1080 cells (WO-A1-9621014), HEK 293, thereby allowing production of recombinant retrovirus that are capable of surviving inactivation by human serum.

Biological Activity

The present invention resides in the use of a ubiquitin promoter to direct *in vivo* expression of therapeutic genes after transfer of such genes to the central nervous system.

Accordingly, in a particular embodiment, the invention is directed to the use of the recombinant expression vector of the invention for the manufacture of a pharmaceutical composition, useful for the treatment of a neurological disease or disorder. The pharmaceutical composition of the invention preferably is a composition suited for injection or implantation into the human brain. Such compositions may be provided in the form of vials of frosen cells, optionally provided in a freeze medium in suspension.

Pharmaceutical compositions suited for injection or implantation may be prepared by the skilled person according to conventional methods (see e.g. Scientia Medicinalis, 1997, Schering AG, ISSN 1433-190X). The pharmaceutical compounds of the present invention may thus be formulated for in ampoules, pre-filled syringes, small volume infusion containers, etc. The compositions may take such forms as suspensions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilising and/or dispersing agents.

In another aspect the invention provides a method for the treatment of a living body suffering from a neurological disease or disorder which is responsive to a therapeutically active polypeptide, which method comprises administering to the living body a retroviral particle obtained by the method of the invention, encoding the therapeutically active polypeptide; or by implanting into the living body, cells of the invention, producing the therapeutically active polypeptide.

The neurological disorders contemplated according to the present invention are e.g. ischemic stroke; angiogenesis; metabolic disease of the brain; axonal injury; spinal cord injury; Alzheimer's disease; Amyothropic Lateral Sclerosis (ALS); Parkinson's disease; Huntington's disease; motor neuron disease; central nervous

system infections; epilepsy; post polio syndrome; mucopolysaccharidoses (MPS), in particular MPS types I to VII; lipidosis, in particular Gaucher's disease; Lesch-Nyhan syndrome; X-linked ADL; metachromatic leukodystrophy; Krabbe's disease; Charcot-Marie-Tooth disease; Fragile X; epilepsy; Down's syndrome; phenylketonuria; degenerative disorders; or mental disorders.

EXAMPLES

The invention is further illustrated with reference to the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

Example 1

Generation of UbC/EGFP expression plasmid and *In vivo* expression of EGFP under the transcriptional control of UbC

For efficient transfection and expression the human UbC promoter was cloned into a modified version of pcDNA3.1/Zeo. The unmodified pcDNA3.1/Zeo is commercial available from Invitogen. The modified version is smaller than the parent vector, since the ampicillin gene (from position 3933 to 5015) and a sequence from position 2838 to 3134 were removed. In this modified version of pcDNA3.1/Zeo the CMV promoter was replaced with the UbC promoter from pTEJ-8 [*Johansen et al*; FEBS Lett. 1990 **267** 289-294] resulting in pUbi1Z, see Fig. 1.

The cDNA for Enhanced Green Fluorescence Protein (EGFP) was cloned into the polylinker of pUbi1Z (EcoRI - XhoI) and this expression plasmid was transfected into the HiB5 cell line (HiB5 is a immortalised neural stem cell from a rat E 14 embryo hipocampus [*Refranz et al*; Cell 1991 **66** 713-729]. The HiB5 cells were transfected with Lipid 6 from Invitrogen according to manufactories recommendations.

Approximately 300.000 cells from a single HiB5/EGFP clone (pUbi/EGFP-11) were transplanted into the striatum of male Wistar rats (≈ 300 g; Moellegard Breeding Centre, Denmark). At different time intervals (see Table 1), the animals were sacrificed, the brain removed, frozen to - 80 °C, and sectioned in 35 µm thick sections. Every second section was sampled and analysed using an Olympus BX50 fluorescent microscope.

The score in Table 1 is the number of sections, in which cells expressing EGFP were detected. The density of the fluorescent was qualitatively graded from 1 to 5, and the grading was summarised for each animal. The study continues after the 88 days, however, data are not yet obtained.

Table 1

Stability of in vivo expression

Days post-transplantation	Number of sections with GFP-cells	Density - score
5	10	37
5	11	37
34	12	14
35	13	30
59	15	29
74	11	21
83	9	11
87	7	9
88	6	6

This *in vivo* study demonstrates that the human UbC promoter direct lasting *in vivo* expression of a transgene.

Example 2

Ubiquitin driven Expression of Therapeutic Genes and *in vivo* Testing in Animal Models

The gene encoding GDNF was cloned using conventional techniques or as described in WO 93/06116 and inserted into the pUbi1Z expression vector. Clones of Immortalised neural stem cells (rat and human) and immortalised cerebral endothelial cells (rat or human) expressing therapeutic genes have been generated.

Clones will be studied in short-term experiments in order to establish the ability of each cell line to secrete the introduced neurotrophic factor (e.g. NGF, GDNF, NTN or CNTF) *in vivo* in sufficiently high amounts to produce full neuroprotective effects

in established models. For NGF secreting cell lines, the cells will be implanted into the septum in rats subjected to a unilateral fimbria-fornix lesion, and the magnitude of cholinergic cell loss in the medial septal nucleus will be established 2 weeks after operation. From previous studies on NGF-secreting rat HiB5 and RBE cells it is known that an implant of 2 x 10^5 cells with an estimated *in vivo* secretion rate of 50 ng NGF/day will be able to provide complete protection in this model.

For GDNF and NTN secreting cell lines, cells will be implanted in the substantia nigra and striatum, first, in C57 mice treated one week later with a single injection of MPTP (40 mg/kg s.c.). The loss of dopamine neurones in the nigra and the reduction in dopamine levels and tyrosine hydroxylase immunoreactivity in the striatum will be analysed 4 weeks after injection. Secondly, cells will be implanted into the substantia nigra in rats subjected to an intrastriatal injection of 6-hydroxydopamine (20 ug) and the magnitude of cell loss in the substantia nigra and the degree of denervation in the striatum will be evaluated at 4 weeks after lesion. In these animals the nigral neurones will be pre-labelled with the retrograde tracer FluoroGold from the striatum according to the procedure established in the Swedish laboratory, in order to provide an accurate estimation of the magnitude of lesion induced nigral cell death. In all cases transplants of cells expressing the reporter gene, EGFP, will be used as controls. Since the minimum number of cells necessary to produce a full neuroprotective effect has not been established for GDNF and NTN, different cell numbers, from 2-8x10^5 cells, will be tested.

CNTF and NGF cells will be evaluated by their protective effect against quinolinic acid lesion (200 nmol) of striatal medium size projection neurones. This type of lesion induces complete neuronal death in 2 days to one week. The cells will be transplanted prior to the lesion and neuroprotection assessed one week post lesion, determining neuroprotection in the striatum, and preservation of innervation of target territories in the SNr.

The long-term functional effects and the stability of transgene expression will be studied in animal models relevant to the three clinical conditions, Parkinson's disease, Huntington´s chorea and dementia. Three animal models will be used to study long-term functional effects of the therapeutic cell lines.

Rats with unilateral intrastriatal 6-hydroxydopamine lesions will receive implants of GDNF or NTN producing cells, either into the substantia nigra, 3 days after

the lesion (i.e. prior to the onset of cell death), or into the striatum, 4 weeks after the lesion (at the time when dopamine neuron degeneration is well advanced). The ability of the cell implants to reverse the Parkinsonian condition in tests of spontaneous motor behaviour (stepping and paw use tests) and to prevent the progressive neuro-degeneration long-term will be studied over a 6 month period.

NGF-secreting human neural and endothelial cells will be implanted into the nucleus basalis and septum in aged cognitively impaired rats (22-24 month old Sprague-Dawley rats) following the protocol used in previous studies on NGF secreting rat HiB5 cells in the Lund laboratory. Groups of young unimpaired rats, transplanted identically, will be studied in parallel. The ability of the cells to reverse the age dependent learning and memory impairment in the water maze test and the age-dependent cholinergic neuronal atrophy in basalis and septum will be studied over 1 and 6 months. EGFP expressing cells will be used as control.

The neuroprotective effects of the NGF or CNTF secreting human cell lines will be studied in rats receiving injections of quinolinic acid into the striatum, as above. The cells will be implanted one week before the lesion, and the ability of the cells to block the excitotoxic striatal damage, as well as to prevent the development of impairments in motor behaviours, will be studied both short-term (4 days and 1 month) and long-term (6 months). Long-term expression of the transgene will be evaluated as above.

Example 3

Generation of an UbC containing ProCon Vector and Production of Retroviral Particles by Packaging Cells

In a preferred embodiment of the invention immortalised cerebral endothelial cells are transformed by a ProCon vector as described above carrying the gene encoding GDNF under transcriptional control of the human ubiquitin promoter.

The vector for transformation of the cerebral endothelial cells may be obtained by insertion of the human ubiquitin promoter prepared by PCR from the plasmid pTEJ-8 described in FEBS Letters, 1990 **267** (2) 289-294, into the polylinker of the ProCon vector described in WO 96/07748.

A GDNF encoding gene may be cloned using conventional techniques or as described in WO 93/06116 and inserted into the body of the ProCon vector.

Retroviral particles are produced by introducing the ProCon vector obtained as above into a packaging cell line followed by isolation of the retroviral particles produced.

Immortalised cerebral endothelial cells may then be infected by the retroviral particles produced by the packaging cell line.

According to another approach, genes encoding GDNF or NGF may be introduced into rat cerebral endothelial cells (RBE) by transfection with an eukaryotic expression vector.

The eukaryotic expression vector may be constructed by insertion of GDNF cDNA or NGF cDNA into the polylinker of the plasmid pTEJ-8 or pUbi1Z.

The resulting vector may be used to transfect immortalised cerebral endothelial cells of rat or human origin.

The genetically transformed immortalised endothelial cells may hereafter be implanted into the CNS of an individual suffering from Parkinson's disease or another disease susceptible to treatment with GDNF.

The immortalised neural stem cells or immortalised endothelial cells may be implanted into the striatum of the host but may also be implanted into other parts of the brain.

As defined herein the term "polylinker" is used for a short stretch of artificially synthesised DNA which carries unique restriction sites allowing the easy insertion of any promoter or DNA segment.

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1578 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(vi) ORIGINAL SOURCE: λHub13 (EMBO J. (1985) 4, 755-759)
(A) ORGANISM: Human
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TCTGCCGAGT CATTGTCCTT GTCCCGCGGC CCCGGGAGCC CCCCGCGACC GGCCTGGGAG 60
GCTCAGGGAG GTTGAAGGGG GCTGAGCAAA GGAAGCCCCG TCATTACCTC AAATGTGACC 120
CAAAAATAAA GACCCGTCCA TCTCGCAGGG TGGGCCAGGG CGGGTCAGGA GGGAGGGGAG 180
GGAGACCCCG ACTCTGCAGA AGGCGCTCGC TGCGTGCCCC ACGTCCGCCG AACGCGGGGT 240
TCGCGACCCG AGGGGACCGC GGGGGCTGAG GGGAGGGGCC GCGGAGCCGC GGCTAAGGAA 300
CGCGGGCCGC CCACCCGCTC CGGGTGCAGC GGCCTCCGCG CCGGGTTTTG GCGCCTCCCG 360
CGGGCGCCCC CCTCCTCACG GCGAGCGCTG CCACGTCAGA CGAAGGGCGC AGCGAGCGTC 420
CTGATCCTTC CGCCCGGACG CTCAGGACAG CGGCCCGCTG CTCATAAGAC TCGGCCTTAG 480
AACCCCAGTA TCAGCAGAAG GACATTTTAG GACGGGACTT GGGTGACTCT AGGGCACTGG 540
TTTTCTTTCC AGAGAGCGGA ACAGGCGAGG AAAAGTAGTC CCTTCTCGGC GATTCTGCGG 600
AGGGATCTCC GTGGGGCGGT GAACGCCGAT GATTATATAA GGACGCGCCG GGTGTGGCAC 660
AGCTAGTTCC GTCGCAGCCG GGATTTGGGT CGCGGTTCTT GTTTGTGGAT CGCTGTGATC 720
GTCACTTGGT GAGTAGCGGG CTGCTGGGCT GGCCGGGGCT TTCGTGGCCG CCGGGCCGCT 780
CGGTGGGCCG GGGCTTTCGT GGCCGCCGGG CCGCTCGGTG GGACGGAAGC GTGTGGAGAG 840
ACCGCCAAGG GCTGTAGTCT GGGTCCGCGA GCAAGGTTGC CCTGAACTGG GGGTTGGGGG 900
GAGCGCAGCA AAATGGCGGC TGTTCCCGAG TCTTGAATGG AAGACGCTTG TGAGGCGGGC 960
TGTGAGGTCG TTGAAACAAG GTGGGGGGCA TGGTGGGCGG CAAGAACCCA AGGTCTTGAG 1020
GCCTTCGCTA ATGCGGGAAA GCTCTTATTC GGGTGAGATG GGCTGGGGCA CCATCTGGGG 1080
ACCCTGACGT GAAGTTTGTC ACTGACTGGA GAACTCGGTT TGTCGTCTGT TGCGGGGGCG 1140
GCAGTTATGG CGGTGCCGTT GGGCAGTGCA CCCGTACCTT TGGGAGCGCG CGCCCTCGTC 1200
GTGTCGTGAC GTCACCCGTT CTGTTGGCTT ATAATGCAGG GTGGGGCCAC CTGCCGGTAG 1260
GTGTGCGGTA GGCTTTTCTC CGTCGCAGGA CGCAGGGTTC GGGCCTAGGG TAGGCTCTCC 1320
TGAATCGACA GGCGCCGGAC CTCTGGTGAG GGGAGGGATA AGTGAGGCGT CAGTTTCTTT 1380
GGTCGGTTTT ATGTACCTAT CTTCTTAAGT AGCTGAAGCT CCGGTTTTGA ACTATGCGCT 1440
CGGGGTTGGC GAGTGTGTTT TGTGAAGTTT TTTAGGCACC TTTTGAAATG TAATCATTTG 1500
GGTCAATATG TAATTTTCAG TGTTAGACTA GTAAATTGTC CGCTAAATTC TGGCCGTTTT 1560
TGGCTTTTTT GTTAGACG 1578

CLAIMS

1. A recombinant expression vector comprising a gene encoding a therapeutically active polypeptide, which gene is under transcriptional control of an ubiquitin promoter.

2. The recombinant expression vector according to claim 1, which is a recombinant viral expression vector selected from the group consisting of retroviral vectors, recombinant adenovirus vectors, recombinant adeno-associated virus vectors, vaccinia virus vectors and recombinant herpes virus vectors.

3. The recombinant viral expression vector according to claim 2, comprising
   (i) a 5'LTR region of the structure U3-R-U5;
   (ii) a packaging signal;
   (iii) at least one nucleotide sequence encoding a therapeutically active polypeptide; and
   (iv) a 3'LTR region of the structure U3-R-U5;
   wherein the nucleotide sequence of step (iii) encoding a therapeutically active polypeptide is under transcriptional control of an ubiquitin promoter.

4. The recombinant viral expression vector according to claim 2, comprising
   (i) a 5'LTR region of the structure U3-R-U5;
   (ii) a packaging signal;
   (iii) at least one nucleotide sequence encoding a therapeutically active polypeptide;
   (iv) a 3'LTR region comprising a completely deleted, or partially deleted U3 region, wherein the deleted U3 region has been replaced by a polylinker sequence comprising an ubiquitin promoter, followed by the R and U5 region;
   wherein the nucleotide sequence of step (iii) encoding the therapeutic gene is under transcriptional control of an ubiquitin promoter.

5. The recombinant expression vector according to any of claims 1-4, wherein the therapeutically active polypeptide encoded by the gene is a protein capable of ameliorating neurological disorders.

6. The recombinant expression vector according to claim 5, wherein the therapeutically active polypeptide encoded by the gene is a Nerve Growth Factor (NGF); a Fibroblast Growth Factor (FGF), in particular an acidic or a basic Fibroblast Growth Factor (aFGF or bFGF); an Insulin-like Growth Factor, in particular IGF I or IGF II; an Endothelial Growth Factor (EGF), in particular a Vascular Endothelial Growth and Permeability Factor (VEGPF); a member of the Transforming Growth Factor (TGF) superfamily, including a Transforming Growth Factor-α and -β (TGFα and TGFβ); a Glial Derived Neurotrophic Factor (GDNF); a Ciliary Neurotrophic Factor (CNTF); a Brain Derived Neurotrophic Factor (BDNF); a Neurotrophin, in particular Neurotrophin 3, 4/5, 6 or 7; a Neuturin (NTN); a Percipin; a Tumor Necrosis Factor (TNF), in particular TNF-α; an interferon, in particular Interferon-α, Interferon-β or Interferon-γ; an interleukin (IL), in particular IL-1, IL-1β, GMCSF, and IL-2 to IL-14; a choline acetyl transferase; a Tyrosine Hydroxylase (TH); a tyrosine decarboxylase; an NT-3 or NT-4; a Super Oxide Dismutase (SOD); an Ornithine Transcarbamylase (OTC); a neurotransmitter; a neuromodulator; a neurohormone; a neurotropic factor; a neuroprotective agent; a negative selective marker such as viral a thymidine kinase, in particular Herpex Simplex thymidine kinase, a cytomegalovirus thymidine kinase, a varicella-zoster virus thymidine kinase, or a cytosine deamidase.

7. The recombinant expression vector according to claim 1, which is an eukaryotic expression vector, e.g. pTEJ-4, pTEJ-8, pUbi12.

8. The recombinant expression vector according to any of claims 1-7, in which the ubiquitin promoter is a human ubiquitin promoter.

9. The recombinant expression vector according to claim 8, in which the ubiquitin promoter is the human ubiquitin C promoter (UbC).

10. The recombinant expression vector according to claim 9, wherein the ubiquitin promoter has the nucleotide sequence presented as SEQ ID NO: 1 in the attached sequence listing.

11. An eukaryotic cell, transfected with the eukaryotic expression vector according to claim 7.

12. An eukaryotic cell according to claim 11, which is an immortalised neural or cerebral endothelial cell.

13. A packaging cell line comprising the retroviral expression vector according to any of claims 2-6, and one or more nucleotide constructs encoding the proteins required for the genome of the retroviral vector to be packaged.

14. A method of producing a retroviral particle by culturing the packaging cell line according to claim 13.

15. The retroviral particle obtained according to claim 14 by culturing the packaging cell line according to claim 13.

16. An eukaryotic cell, e.g. an immortalised cerebral endothelial cell line, infected by a retroviral particle obtained by the method of claim 11.

17. Use of the recombinant expression vector according to any of claims 1-10 for the manufacture of a pharmaceutical composition, useful for the treatment of a neurological disease or disorder.

18. The use according to claim 17, in which the pharmaceutical composition is a composition suited for injection or implantation into the human brain.

19. The use according to claim 18, in which the pharmaceutical composition is a cell line, provided in provided in the form of frozen cells, optionally provided in a suspension in a freeze medium.

20. The use according to any of claims 17-19, in which the neurological disorder is ischemic stroke; angiogenesis; metabolic disease of the brain; axonal injury; spinal cord injury; Alzheimer's disease; Amyothropic Lateral Sclerosis (ALS); Parkinson's disease; Huntington's disease; a motor neuron disease; a central nervous system infections; epilepsy; post polio syndrome; mucopolysaccharidoses (MPS), in particular MPS types I to VII; lipidosis, in particular Gaucher's disease; Lesch-Nyhan syndrome; X-linked ADL; metachromatic leukodystrophy; Krabbe's disease; Charcot-Marie-Tooth disease; Fragile X; epilepsy; Down's syndrome; phenylketonuria; a degenerative disorder; or a mental disorder.

21. A method for the treatment of a living body suffering from a neurological disease or disorder which is responsive to a therapeutically active polypeptide, which method comprises:

(i) administering to the living body, a retroviral particle obtained by the method according to claim 13, encoding the therapeutically active polypeptide; or

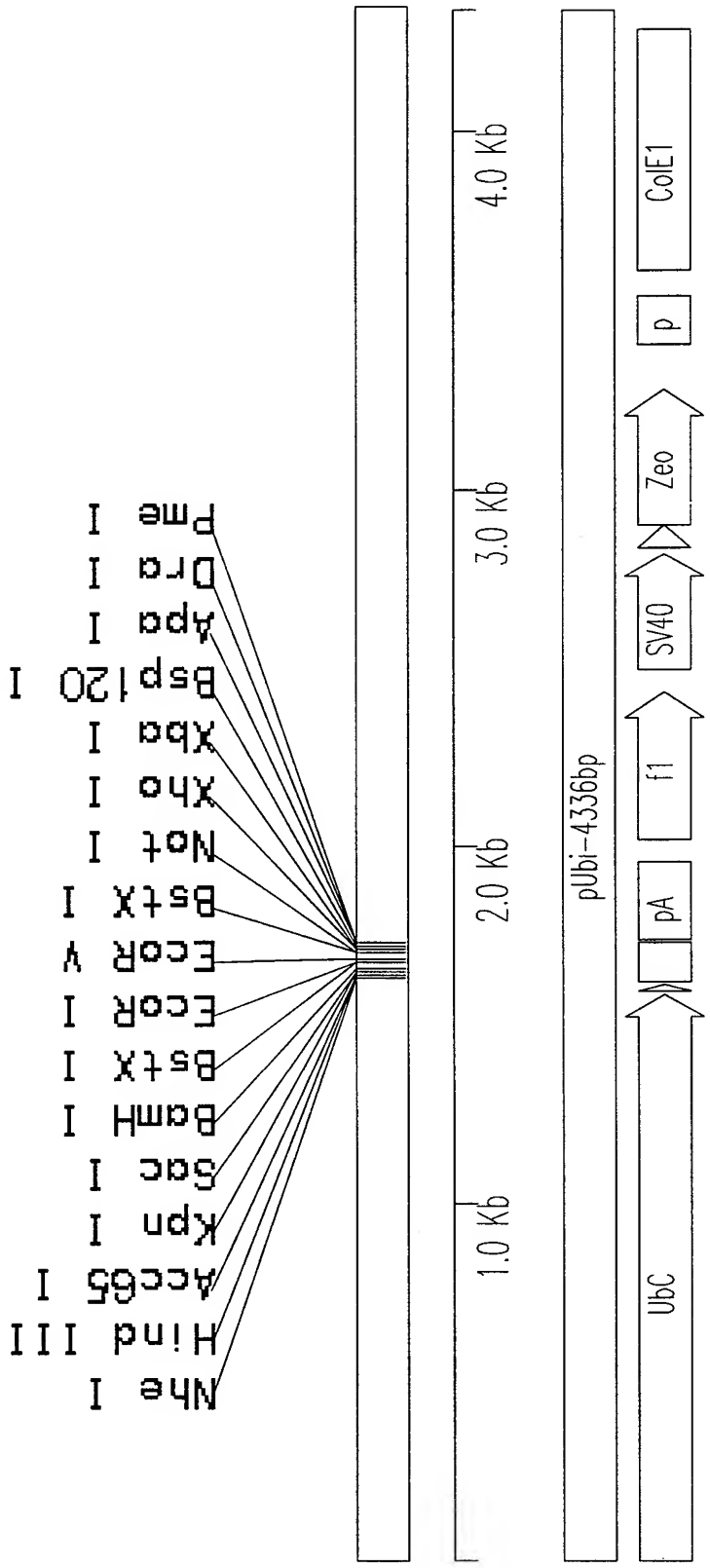
(ii) implanting into the living body, cells according to any of claims 10-12 producing the therapeutically active polypeptide.

22. The method according to claim 21, in which the living body is a human suffering from a neurological disease or disorder which is responsive to the therapeutically active polypeptide encoded by the gene according to any of claims 1-10.

23. The method according to claim 22, in which the neurological disease or disorder is ischemic stroke; angiogenesis; metabolic disease of the brain; axonal injury; spinal cord injury; Alzheimer's disease; Amyothropic Lateral Sclerosis (ALS); Parkinson's disease; Huntington's disease; a motor neuron disease; a central nervous system infections; epilepsy; post polio syndrome;

mucopolysaccharidoses (MPS), in particular MPS types I to VII; lipidosis, in particular Gaucher's disease; Lesch-Nyhan syndrome; X-linked ADL; metachromatic leukodystrophy; Krabbe's disease; Charcot-Marie-Tooth disease; Fragile X; epilepsy; Down's syndrome; phenylketonuria; a degenerative disorder; or a mental disorder.

Fig. 1


Nhe I
Hind III
Acc65 I
Kpn I
Sac I
BamH I
BstX I
EcoR I
EcoR V
BstX I
Not I
Xho I
Xba I
Bsp120 I
Apa I
Dra I
Pme I
1.0 Kb
2.0 Kb
3.0 Kb
4.0 Kb
pUbi-4336bp
UbC
pA
f1
SV40
Zeo
p
ColE1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 98/00037

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N5/16 C12N5/22 C12N7/01 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 97 15664 A (THOMAE GMBH DR K ;ENENKEL BARBARA (DE); GANNON FRANK (DE); BERGEMA) 1 May 1997 see the whole document ---	1-7, 11-17,20
X	EP 0 342 926 A (LUBRIZOL GENETICS INC) 23 November 1989 see the whole document ---	1,7,11, 16
X	JOHANSEN TE ET AL: "Biosynthesis of peptide precursors and protease inhibitors using new constitutive and inducible eukaryotic expression vectors." FEBS LETT, JUL 16 1990, 267 (2) P289-94, NETHERLANDS, XP002069981	1,2, 5-12,16
Y	see the whole document --- -/--	1-23

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 July 1998	10. 07. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hillenbrand, G

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 98/00037

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHORPP, M. ET AL.: "The human ubiquitin C promoter directs high ubiquitous expression of transgenes in mice" NUCLEIC ACIDS RESEARCH, vol. 24, no. 9, 1996, pages 1787-1788, XP000001788 cited in the application	1,2, 7-12,16
Y	see the whole document	1-23

Y	NENOI,M. ET AL.: "Heterogeneous structure of the polyubiquitin gene UbC of HeLa S3 cells" GENE, vol. 175, 1996, pages 179-185, XP004043312 see the whole document	1-23

Y	WO 96 07748 A (GSF FORSCHUNGSZENTRUM UMWELT ;GUENZBURG WALTER HENRY (DE); SALLER) 14 March 1996 cited in the application see the whole document	1-23

Y	WO 95 09654 A (US HEALTH) 13 April 1995 cited in the application see the whole document	1-23

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00037

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 21-23 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr. ...nal Application No
PCT/DK 98/00037

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